METHODS FOR GENERATING HYPERMUTABLE MICROBES

This application claims the benefit of provisional application S.N. 60/181,929 filed February 11, 2000.

FIELD OF THE INVENTION

The invention is related to the area of mismatch repair genes. In particular it is related to the field of *in situ* mutagenesis of single celled organisms.

BACKGROUND OF THE INVENTION

Within the past four years, the genetic cause of the Hereditary Nonpolyposis Colorectal Cancer Syndrome (HNPCC), also known as Lynch syndrome II, has been ascertained for the majority of kindred's affected with the disease (14). The molecular basis of HNPCC involves genetic instability resulting from defective mismatch repair (MMR). Several genes have been identified in humans that encode for proteins and appear to participate in the MMR process, including the *mutS* homologs *GTBP, hMSH2,* and *hMSH3* and the *mutL* homologs *hMLH1*, *hMLH3*, *hPMS1*, and *hPMS2* (4,9,11,17,19,22,24,38). Germline mutations in four of these genes (*hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*) have been identified in HNPCC kindred's (13). Though the mutator defect that arises from the MMR deficiency can affect any DNA sequence, microsatellite sequences are particularly sensitive to MMR abnormalities (14, 25, 27, 29). Microsatellite instability (MI) is therefore a useful indicator of defective MMR. In addition to its occurrence in virtually all tumors arising in HNPCC patients, MI is found in a small fraction of sporadic tumors with distinctive molecular and phenotypic properties (13).

HNPCC is inherited in an autosomal dominant fashion, so that the normal cells of affected family members contain one mutant allele of the

relevant MMR gene (inherited from an affected parent) and one wild-type allele (inherited from the unaffected parent). During the early stages of tumor development, however, the wild-type allele is inactivated through a somatic mutation, leaving the cell with no functional MMR gene and resulting in a profound defect in MMR activity. Because a somatic mutation in addition to a germ-line mutation is required to generate defective MMR in the tumor cells, this mechanism is generally referred to as one involving two hits, analogous to the biallelic inactivation of tumor suppressor genes that initiate other hereditary cancers. In line with this two-hit mechanism, the non-neoplastic cells of HNPCC patients generally retain near normal levels of MMR activity due to the presence of the wild-type allele (11, 13, 24). In addition, similar findings are observed in other diploid organisms (2, 5, 8).

The ability to alter signal transduction pathways by manipulation of a gene product's function, either by over-expression of the wild type protein or a fragment thereof, or by introduction of mutations into specific protein domains of the protein, the so-called dominant-negative inhibitory mutant, were described over a decade ago in the yeast system *Saccharomyces cerevisiae* by Herskowitz (Nature 329:219-222, 1987). It has been demonstrated that over-expression of wild type gene products can result in a similar, dominant-negative inhibitory phenotype due most likely to the "saturating-out" of a factor, such as a protein, that is present at low levels and necessary for activity; removal of the protein by binding to a high level of its cognate partner results in the same net effect, leading to inactivation of the protein and the associated signal transduction pathway.

Recently, work done by Nicolaides et.al. (*32*) has demonstrated the utility of introducing dominant negative inhibitory mismatch repair mutants into mammalian cells to confer global DNA hypermutability. There is a need in the art for additional techniques for generating mutations in bacteria which can be used to make strains for production, biocatalysis, bioremediation, and drug discovery.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for rendering bacterial cells hypermutable.

It is another object of the present invention to provide genetically altered bacteria.

It is yet another object of the present invention to provide a method to produce bacteria that are hypermutable.

It is an object of the invention to provide a method to inactivate the process that results in hypermutable cells following strain selection.

It is a further object of the invention to provide a method of mutating a gene of interest in a bacterium.

These and other embodiments of the invention are provided by one or more of the embodiments described below. In one embodiment, a method is provided for making a hypermutable bacteria. A polynucleotide comprising a dominant negative allele of a mismatch repair gene is introduced into a bacterium, whereby the cell becomes hypermutable. Preferably the allele is under the control of an inducible transcription regulatory sequence.

According to another aspect of the invention a homogeneous composition of cultured, hypermutable, bacteria is provided. The bacteria comprise a dominant negative allele of a mismatch repair gene. Preferably the allele is under the control of an inducible transcription regulatory sequence.

Another embodiment of the invention provides a method for generating a mutation in a gene of interest. A bacterial culture comprising the gene of interest and a dominant negative allele of a mismatch repair gene is grown. The cell is hypermutable. It is tested to determine whether the gene of interest harbors a mutation. Preferably the allele is under the control of an inducible transcription regulatory sequence.

According to still another aspect of the invention a method for generating a mutation in a gene of interest is provided. A bacterium comprising the gene of interest and a dominant negative allele of a mismatch repair gene is grown to form a population of mutated bacteria. The population of mutated bacteria is cultivated under trait selection conditions. At least one of the cultivated bacteria is tested to determine that the gene of interest harbors a mutation. Preferably the allele is under the control of an inducible transcription regulatory sequence.

Still another aspect of the invention is a method for enhancing the mutation rate of a bacterium. A bacterium comprising a dominant negative allele of an MMR gene is exposed to a mutagen whereby the mutation rate of the bacterium is enhanced in excess of the rate in the absence of mutagen and in excess of the rate in the absence of the dominant negative allele. Preferably the allele is under the control of an inducible transcription regulatory sequence.

Yet another aspect of the invention is a method for generating an MMR-proficient bacterium with a new output trait. A mismatch repair deficient bacterium comprising a gene of interest and a dominant negative allele of a mismatch repair gene is grown to form a population of mutated bacteria. The population of mutated bacteria is cultivated under trait selection conditions. At least one of the cultivated bacteria is tested to determine that the gene of interest harbors a mutation. Mismatch repair activity is restored to the at least one of the cultivated

bacteria. Preferably the allele is under the control of an inducible transcription regulatory sequence.

These and other embodiments of the invention provide the art with methods that can generate enhanced mutability in bacteria as well as providing prokaryotic organisms harboring potentially useful mutations to generate novel output traits for commercial applications. The ability to create hypermutable organisms using dominant negative alleles has great commercial value for the generation of innovative bacterial strains that display new output features useful for a variety of applications, including but not limited to the manufacturing industry for the generation of new biochemicals useful for detoxifying noxious chemicals from by-products of manufacturing processes or those used as catalysts, as well as helping in remediation of toxins present in the environment, including but not limited to polychlorobenzenes (PCBs), heavy metals and other environmental hazards for which there is a need to remove them from the environment. In addition to obtaining organisms that are useful for removal of toxins from the environment, novel microbes can be selected for enhanced activity to either produce increased quantity or quality of a protein or non-protein therapeutic molecule by means of biotransformation (3).

Biotransformation is the enzymatic conversion, by a microbe or an extract derived from the microbe, of one chemical intermediate to the next product. There are many examples of biotransformation in use for the commercial manufacturing of important biological and chemical products, including Penicillin G, Erythromycin, and Clavulanic Acid as well as organisms that are efficient at conversion of "raw" materials to advanced intermediates and/or final products (Berry, A. Trends Biotechnol. 14(7):250-256). The ability to control DNA hypermutability in host bacterial strains using a dominant negative MMR (as described above) allows for the generation of variant subtypes that can be selected for new phenotypes of commercial interest, including but not limited to organisms that are toxin-resistant, have

the capacity to degrade a toxin *in situ* or the ability to convert a molecule from an intermediate to either an advanced intermediate or a final product. Other applications using dominant negative MMR genes to produce genetic alteration of bacterial hosts for new output traits include but are not limited to recombinant production strains that produce higher quantities of a recombinant polypeptide as well as the use of altered endogenous genes that can transform chemical or catalyze manufacturing downstream processes.

This application teaches of the use of a regulatable dominant negative MMR phenotype to produce a prokaryotic strain with a commercially beneficial output trait. Using this process, microbes expressing a dominant negative MMR can be directly selected for the phenotype of interest. Once a selected bacterium with a specified output trait is isolated, the hypermutable activity of the dominant negative MMR allele can be turned-off by several methods well known to those skilled in the art. For example, if the dominant-negative allele is expressed by an inducible promoter system, including but not limited to promoters such as: TAC-LACI, tryp (Brosius et.al. Gene 27:161-172, 1984), araBAD (Guzman et.al., *J. Bact.* 177:4121-4130, 1995) pLex (La Vallie et.al., Bio.Technology 11:187-193, 1992), pRSET (Schoepfer, R. *Gene* 124:83-85, 1993) , pT7 (Studier *J. Mol. Biol.* 219(1):37-44, 1991) etc., the inducer is removed and the promoter activity is reduced, or a system that excises the MMR gene insert from the host cells harboring the expression vector such as the Cre-lox (Hasan, N. et.al. *Gene* 2:51-56, 1994), as well as methods that can homologously knockout of the expression vector. In addition to the recombinant methods outlined above that have the capacity to eliminate the MMR activity from the microbe, it has been demonstrated that many chemicals have the ability to "cure" microbial cells of plasmids. For example, chemical treatment of cells with drugs including bleomycin (Attfield et al. *Antimicrob. Agents Chemother.* 27:985-988, 1985) or

novobiocin, coumermycin, and quinolones (Fu et al. *Chemotherapy* 34:415-418, 1988) have been shown to result in microbial cells that lack endogenous plasmid as evidenced by Southern analysis of cured cells as well as sensitivity to the appropriate antibiotic (1, 41-43). Whether by use of recombinant means or treatment of cells with chemicals, removal of the MMR-expression plasmid results in the re-establishment of a genetically stable microbial cell-line. Therefore, the restoration of MMR allows host bacteria to function normally to repair DNA. The newly generated mutant bacterial strain that exhibits a novel, selected output trait is now suitable for a wide range of commercial processes or for gene/protein discovery to identify new biomolecules that are involved in generating a particular output trait.

While it has been documented that MMR deficiency can lead to as much as a 1000-fold increase in the endogenous DNA mutation rate of a host, there is no assurance that MMR deficiency alone will be sufficient to alter every gene within the DNA of the host bacterium to create altered biochemicals with new activity(s). Therefore, the use of chemical agents and their respective analogues such as ethidium bromide, EMS, MNNG, MNU, Tamoxifen, 8-Hydroxyguanine, as well as others listed but not limited to in publications by: Khromov-Borisov, N.N., et.al. (Mutat. Res. 430:55-74, 1999); Ohe, T., et.al. (Mutat. Res. 429:189-199, 1999); Hour, T.C. et.al. (Food Chem. Toxicol. 37:569-579, 1999); Hrelia, P., et.al. (Chem. Biol. Interact. 118:99-111, 1999); Garganta, F., et.al. (Environ. Mol. Mutagen. 33:75-85, 1999); Ukawa-Ishikawa S., et.al. (Mutat. Res. 412:99-107, 1998); www.ehs.utah.edu/ohh/mutagens, etc. can be used to further enhance the spectrum of mutations and increase the likelihood of obtaining alterations in one or more genes that can in turn generate host bacteria with a desired new output trait(s) (10, 39, 40). Prior art teaches that mismatch repair deficiency leads to hosts with an increased resistance to toxicity by chemicals with DNA damaging activity. This feature allows

for the creation of additional genetically diverse hosts when mismatch defective bacteria are exposed to such agents, which would be otherwise impossible due to the toxic effects of such chemical mutagens [Colella, G., et.al. (Br. J. Cancer 80:338-343, 1999); Moreland, N.J., et.al. (Cancer Res. 59:2102-2106, 1999); Humbert, O., et.al. (Carcinogenesis 20:205-214, 1999); Glaab, W.E., et.al. (Mutat. Res. 398:197-207, 1998)]. Moreover, prior art teaches that mismatch repair is responsible for repairing chemical-induced DNA adducts, so therefore blocking this process could theoretically increase the number, types, mutation rate and genomic alterations of a bacterial host [Rasmussen, L.J. et.al. (Carcinogenesis 17:2085-2088, 1996); Sledziewska-Gojska, E., et.al. (Mutat. Res. 383:31-37, 1997); and Janion, C. et.al. (Mutat. Res. 210:15-22, 1989)]. In addition to the chemicals listed above, other types of DNA mutagens include ionizing radiation and UV-irradiation, which are known to cause DNA mutagenesis in bacteria can also be used to potentially enhance this process. These agents which are extremely toxic to host cells and therefore result in a decrease in the actual pool size of altered bacterial cells are more tolerated in MMR defective hosts and in turn allow for a enriched spectrum and degree of genomic mutation (7).

This application teaches new uses of MMR deficient bacterial cells to create commercially viable microbes that express novel output traits. Moreover, this application teaches the use of dominant negative MMR genes to decrease the endogenous MMR activity of the host followed by placing the cells under selection to obtain a desired, sought after output trait for commercial applications such as but not limited to recombinant manufacturing, biotransformation and bioremediation. Furthermore, the application teaches the use of restoring MMR activity to the hypermutable bacterial host following strain selection of the variant of interest as a means to genetically "fix" the new mutations in the host genome. The application also teaches the use of enhanced hypermutability in bacteria by using

MMR deficiency and chemical or radiation mutagenesis to create variant subtypes of bacteria useful for commercial and other applications. The application describes uses of hypermutable bacteria for producing strains that can be used to generate new output traits for chemical manufacturing, pharmaceutical and other commercially applicable processes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Western blot of steady-state PMS134 levels in IPTG-treated samples in DH10B strain. Cells containing the pTACPMS134 (lane 2) showed a robust steady state level of protein after induction in contrast to cells expressing empty vector (lane 1). Blots were probed with an anti-human-PMS2 polyclonal antibody.

Figure 2. Western blot of PMS134V5 and PMSR3V5 in IPTG-treated (+) and untreated (-) samples in BL21 strain. Blots were probed with an anti-V5 antibody, which is directed to the C-terminal tag of each protein.

Figure 3. Number of Kanamycin resistant PMS134 and vector control DH10B clones. IPTG-induced strains were grown and plated onto KAN plates and grown for 18 hours at 37°C to identify number of KAN resistant clones due to genetic alteration.

Figure 4. Number of Kanamycin Resistant PMS134, PMSR3 and vector control BL21 clones. IPTG-induced strains were grown and plated onto AMP and KAN plates and grown for 18 hours at 37°C to identify number of KAN resistant clones due to genetic alteration.

Figure 5. (A) Western blot of steady-state ATPMS134flag in IPTG-treated samples in DH10B. Lysates from untransfected cells (lane 1) and a

bacterial clone expressing the Arabidopsis thaliana PMS134 truncated protein with a FLAG epitope fused to the C-terminus (ATPMS134flag) (lane 2) were electrophoresed on SDS-PAGE gels. Blots were probed with an anti-FLAG monoclonal antibody directed to the FLAG epitope. (B) Number of Kanamycin Resistant ATPMS134flag and vector control DH10B clones. IPTG-induced strains were grown and plated onto AMP and KAN plates and grown for an additional 18 hours at 37°C to identify number of KAN resistant clones due to genetic alteration.

Figure 6. Generation of high recombinant producer BGAL-MOR lines in PMS134 expressing DH5alpha host strains.

DETAILED DESCRIPTION OF THE INVENTION

The inventors present a method for developing hypermutable bacteria by altering the activity of endogenous mismatch repair activity of hosts. Wild type and some dominant negative alleles of mismatch repair genes, when introduced and expressed in bacteria, increase the rate of spontaneous mutations by reducing the effectiveness of the endogenous MMR-mediated DNA repair activity, thereby rendering the bacteria highly susceptible to genetic alterations due to hypermutability. Hypermutable bacteria can then be utilized to screen for novel mutations in a gene or a set of genes that produce variant siblings that exhibit a new output trait(s) not found in the wild type cells.

The process of mismatch repair, also called mismatch proofreading, is an evolutionarily highly conserved process that is carried out by protein complexes described in cells as disparate as prokaryotic cells such as bacteria to more complex mammalian cells (14, 29, 31, 33, 34). A mismatch repair gene is a gene that encodes one of the proteins of such a mismatch repair complex. Although not wanting to be bound by any

particular theory of mechanism of action, a mismatch repair complex is believed to detect distortions of the DNA helix resulting from non-complementary pairing of nucleotide bases. The non-complementary base on the newer DNA strand is excised, and the excised base is replaced with the appropriate base that is complementary to the older DNA strand. In this way, cells eliminate many mutations that occur as a result of mistakes in DNA replication, resulting in genetic stability of the sibling cells derived from the parental cell.

Some wild type alleles as well as dominant negative alleles cause a mismatch repair defective phenotype even in the presence of a wild-type allele in the same cell. An example of a dominant negative allele of a mismatch repair gene is the human gene *hPMS2-134*, which carries a truncation mutation at codon 134 (32). The mutation causes the product of this gene to abnormally terminate at the position of the 134th amino acid, resulting in a shortened polypeptide containing the N-terminal 133 amino acids. Such a mutation causes an increase in the rate of mutations, which accumulate in cells after DNA replication. Expression of a dominant negative allele of a mismatch repair gene results in impairment of mismatch repair activity, even in the presence of the wild-type allele. Any mismatch repair allele, which produces such effect, can be used in this invention. In addition, the use of over-expressed wildtype MMR gene alleles from human, mouse, plants, and yeast in bacteria has been shown to cause a dominant negative effect on the bacterial hosts MMR activity (9, 33, 34, 38).

Dominant negative alleles of a mismatch repair gene can be obtained from the cells of humans, animals, yeast, bacteria, plants or other organisms. Screening cells for defective mismatch repair activity can identify such alleles. Mismatch repair genes may be mutant or wild type. Bacterial host MMR may be mutated or not. The term bacteria used in this application include any organism from the prokaryotic *kingdom*. These

organisms include genera such as but not limited to *Agrobacterium, Anaerobacter, Aquabacterium, Azorhizobium, Bacillus, Bradyrhizobium, Cryobacterium, Escherichia, Enterococcus, Heliobacterium, Klebsiella, Lactobacillus, Methanococcus, Methanothermobacter, Micrococcus, Mycobacterium, Oceanomonas, Pseudomonas, Rhizobium, Staphylococcus, Streptococcus, Streptomyces, Thermusaquaticus, Thermaerobacter, Thermobacillus, etc.* Other procaryotes that can be used for this application are listed at (www.bacterio.cict.fr/validgenericnames). Bacteria exposed to chemical mutagens or radiation exposure can be screened for defective mismatch repair. Genomic DNA, cDNA, or mRNA from any cell encoding a mismatch repair protein can be analyzed for variations from the wild type sequence. Dominant negative alleles of a mismatch repair gene can also be created artificially, for example, by producing variants of the *hPMS2-134* allele or other mismatch repair genes (32). Various techniques of site-directed mutagenesis can be used. The suitability of such alleles, whether natural or artificial, for use in generating hypermutable bacteria can be evaluated by testing the mismatch repair activity (using methods described in ref 32) caused by the allele in the presence of one or more wild-type alleles, to determine if it is a dominant negative allele.

A bacterium that over-expresses a wild type mismatch repair allele or a dominant negative allele of a mismatch repair gene will become hypermutable. This means that the spontaneous mutation rate of such bacteria is elevated compared to bacteria without such alleles. The degree of elevation of the spontaneous mutation rate can be at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000-fold that of the normal bacteria as measured as a function of bacterial doubling/minute.

According to one aspect of the invention, a polynucleotide encoding either a wild type or a dominant negative form of a mismatch repair protein is introduced into bacteria. The gene can be any dominant negative allele

encoding a protein which is part of a mismatch repair complex, for example, *mutS, mutL, mutH, or mutY* homologs of the bacterial, yeast, plant or mammalian genes (14, 28). The dominant negative allele can be naturally occurring or made in the laboratory. The polynucleotide can be in the form of genomic DNA, cDNA, RNA, or a chemically synthesized polynucleotide or polypeptide. The molecule can be introduced into the cell by transfection, transformation, conjugation, fusion, or other methods well described in the literature.

Any process can be used whereby a polynucleotide or polypeptide is introduced into a cell. The process of gene transfer can be carried out in a bacterial culture using a suspension culture. The bacteria can be any type classified under the prokaryotes.

In general, gene transfer will be carried out using a suspension of cells but other methods can also be employed as long as a sufficient fraction of the treated cells incorporate the polynucleotide or polypeptide so as to allow recipient cells to be grown and utilized. The protein product of the polynucleotide may be transiently or stably expressed in the cell. Techniques for gene transfer are well known to those skilled in the art. Available techniques to introduce a polynucleotide or polypeptide into a prokaryote include but are not limited to electroporation, transduction, cell fusion, the use of chemically competent cells (e.g. calcium chloride), and packaging of the polynucleotide together with lipid for fusion with the cells of interest. Once a cell has been transformed with the dominant negative mismatch repair gene or protein, the cell can be propagated and manipulated in either liquid culture or on a solid agar matrix, such as a petri dish. If the transfected cell is stable, the gene will be retained and expressed at a consistent level when the promoter is constitutively active, or when in the presence of appropriate inducer molecules when the promoter is inducible, for many cell generations, and a stable, hypermutable bacterial strain results.

An isolated bacterial cell is a clone obtained from a pool of a bacterial culture by chemically selecting out non-recipient strains using, for example, antibiotic selection of an expression vector. If the bacterial cell is derived from a single cell, it is defined as a clone.

A polynucleotide encoding a dominant negative form of a mismatch repair protein can be introduced into the genome of a bacterium or propagated on an extra-chromosomal plasmid. Selection of clones harboring the mismatch repair gene expression vector can be accomplished by addition of any of several different antibiotics, including but not limited to ampicillin, kanamycin, chloramphenicol, zeocin, and tetracycline. The microbe can be any species for which suitable techniques are available to produce transgenic microorganisms, such as but not limited to genera including *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Escherichia* and others.

Any method for making transgenic bacteria known in the art can be used. According to one process of producing a transgenic microorganism, the polynucleotide is transfected into the microbe by one of the methods well known to those in the art. Next, the microbial culture is grown under conditions that select for cells in which the polynucleotide encoding the mismatch repair gene is either incorporated into the host genome as a stable entity or propagated on a self-replicating extra-chromosomal plasmid, and the protein encoded by the polynucleotide fragment transcribed and subsequently translated into a functional protein within the cell. Once transgenic microbe is engineered to harbor the expression construct, it is then propagated to generate and sustain a culture of transgenic microbes indefinitely.

Once a stable, transgenic microorganism has been engineered to express a functional mismatch repair (MMR) protein, the microbe can be exploited to create novel mutations in one or more target gene(s) of interest harbored within the same microorganism. A gene of interest can be any gene naturally possessed by the bacterium or one introduced into the

bacterial host by standard recombinant DNA techniques. The target gene(s) may be known prior to the selection or unknown. One advantage of employing such transgenic microbes to induce mutations in resident or extra-chromosomal genes within the microbe is that it is unnecessary to expose the microorganism to mutagenic insult, whether it be chemical or radiation in nature, to produce a series of random gene alterations in the target gene(s). This is due to the highly efficient nature and the spectrum of naturally occurring mutations that result as a consequence of the altered mismatch repair process. However, it is possible to increase the spectrum and frequency of mutations by the concomitant use of either chemicals and/or radiation together with MMR defective cells. These include DNA mutagens, DNA alkylating agents, DNA intercalating agents, DNA oxidizing agents, ionizing raditation, and ultraviolet radiation. The net effect of the combination treatment is the increase in altered gene pool in the genetically altered microbe that result in an increased alteration of an allele(s) that are useful for producing new output traits. Another benefit of using MMR-defective microbes that are taught in this application is that one can perform a genetic screen for the direct selection of variant sub-clones that exhibit new output traits with commercially important applications. This allows one to bypass tedious and time consuming gene identification, isolation and characterization.

Mutations can be detected by analyzing the recombinant microbe for alterations in the genotype and/or phenotype post-activation of the decreased mismatch repair activity of the transgenic microorganism. Novel genes that produce altered phenotypes in MMR-defective microbial cells can be discerned by any variety of molecular techniques well known to those in the art. For example, the microbial genome can be isolated and a library of restriction fragments cloned into a plasmid vector. The library can be introduced into a "normal" cell and the cells exhibiting the novel phenotype screened. A plasmid is isolated from those normal cells that

exhibit the novel phenotype and the gene(s) characterized by DNA sequence analysis. Alternatively, differential messenger RNA screen can be employed utilizing driver and tester RNA (derived from wild type and novel mutant respectively) followed by cloning the differential transcripts and characterizing them by standard molecular biology methods well known to those skilled in the art. Furthermore, if the mutant sought is on encoded by an extrachromosmal plasmid, then following co-expression of the dominant negative MMR gene and the gene of interest to be altered and phenotypic selection, the plasmid is isolated from mutant clones and analyzed by DNA sequence analysis by methods well known to those in the art. Phenotypic screening for output traits in MMR-defective mutants can be by biochemical activity and/or a physical phenotype of the altered gene product. A mutant phenotype can also be detected by identifying alterations in electrophoretic mobility, DNA binding in the case of transcription factors, spectroscopic properties such as IR, CD, X-ray crystallography or high field NMR analysis, or other physical or structural characteristics of a protein encoded by a mutant gene. It is also possible to screen for altered novel function of a protein *in situ*, in isolated form, or in model systems. One can screen for alteration of any property of the microorganism associated with the function of the gene of interest, whether the gene is known prior to the selection or unknown. The aforementioned screening and selection discussion is meant to illustrate the potential means of obtaining novel mutants with commercially valuable output traits.

Plasmid expression vectors that harbor the mismatch repair (MMR) gene inserts can be used in combination with a number of commercially available regulatory sequences to control both the temporal and quantitative biochemical expression level of the dominant negative MMR protein. The regulatory sequences can be comprised of a promoter, enhancer or promoter/enhancer combination and can be inserted either upstream or downstream of the MMR gene to control the expression level. The

regulatory promoter sequence can be any of those well known to those in the art, including but not limited to the lacI, tetracycline, tryptophan-inducible, phosphate inducible, T7-polymerase-inducible (30), and steroid inducible constructs as well as sequences which can result in the excision of the dominant negative mismatch repair gene such as those of the Cre-Lox system. These types of regulatory systems are familiar to those skilled in the art.

Once a microorganism with a novel, desired output trait of interest is created, the activity of the aberrant MMR activity can beattenuated or eliminated by any of a variety of methods, including removal of the inducer from the culture medium that is responsible for promoter activation, gene disruption of the aberrant MMR gene constructs, electroporation and/or chemical curing of the expression plasmids (Brosius, Biotechnology 10:205-225,1988; Wang et al., J. of Fujian Agricultural University 28:43-46,1999; Fu et. al., Chem Abstracts 34:415-418, 1988). The resulting microbe is now useful as a stable strain that can be applied to various commercial applications, depending upon the selection process placed upon it.

In cases where genetically deficient mismatch repair bacteria [strains such as but not limited to: M1 (mutS) and in EC2416 (mutS delta umuDC), and mutL or mutY strains] are used to derive new output traits, transgenic constructs can be used that express wild-type mismatch repair genes sufficient to complement the genetic defect and therefore restore mismatch repair activity of the host after trait selection [Grzesiuk, E. et.al. (Mutagenesis 13:127-132, 1998); Bridges, B.A., et.al. (EMBO J. 16:3349-3356, 1997); LeClerc, J.E., Science 15:1208-1211, 1996); Jaworski, A. et.al. (Proc. Natl. Acad. Sci USA 92:11019-11023, 1995)]. The resulting microbe is genetically stable and can be applied to various commercial practices.

The use of over expressing foreign mismatch repair genes from human and yeast such as PMS1, MSH2, MLH1, MLH3, etc. have been previously demonstrated to produce a dominant negative mutator phenotype in bacterial hosts (35, 36, 37). In addition, the use of bacterial strains expressing prokaryotic dominant negative MMR genes as well as hosts that have genomic defects in endogenous MMR proteins have also been previously shown to result in a dominant negative mutator phenotype (29,32). However, the findings disclosed here teach the use of MMR genes, including the human PMSR2 and PMSR3 gene (ref 19), the related PMS134 truncated MMR gene (ref 32), the plant mismatch repair genes **(SARAH include Plant patent application)** and those genes that are homologous to the 134 N-terminal amino acids of the PMS2 gene which include the MutL family of MMR proteins and including the PMSR and PMS2L homologs desribed by Hori et.al. (accession number NM_005394 and NM_005395) and Nicolaides (reference 19) to create hypermutable microbes. In addition, this application teaches the use of DNA mutagens in combination with MMR defective microbial hosts to enhance the hypermutable production of genetic alterations. This accentuates MMR activity for generation of microorganisms with commercially relevant output traits such as but not limited to recombinant protein production strains, biotransformation, and bioremediation.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples that will be provided herein for purposes of illustration only, and are not intended to limit the scope of the invention

EXAMPLES

Example 1: Generation of inducible MMR dominant negative allele vectors

Bacterial expression constructs were prepared to determine if the human PMS2 related gene (hPMSR3) (19) and the human PMS134 gene (32) are capable of inactivating the bacterial MMR activity and thereby increase the overall frequency of genomic hypermutation, a consequence of which is the generation of variant sib cells with novel output traits following host selection. Moreover, the use of regulatable expression vectors will allow for suppression of dominant negative MMR alleles and restoration of the MMR pathway and genetic stability in hosts cells (43). For these studies, a plasmid encoding the hPMS134 cDNA was altered by polymerase chain reaction (PCR). The 5' oligonucleotide has the following structure: 5'-ACG CAT ATG GAG CGA GCT GAG AGC TCG AGT-3' that includes the NdeI restriction site CAT ATG. The 3'-oligonucleotide has the following structure: 5'-GAA TTC TTA TCA CGT AGA ATC GAG ACC GAG GAG AGG GTT AGG GAT AGG CTT ACC AGT TCC AAC CTT CGC CGA TGC-3' that includes an EcoRI site GAA TTC and the 14 amino acid epitope for the V5 antibody. The oligonucleotides were used for PCR under standard conditions that included 25 cycles of PCR (95^0C for 1 minute, 55^0C for 1 minute, 72^0C for 1.5 minutes for 25 cycles followed by 3 minutes at 72^0C). The PCR fragment was purified by gel electrophoresis and cloned into pTA2.1 (InVitrogen) by standard cloning methods (Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Edition, 2001), creating the plasmid pTA2.1-hPMS134. pTA2.1-hPMS134 was digested with the restriction enzyme EcoRI to release the insert (there are two EcoRI restriction sites in the multiple cloning site of pTA2.1 that flank the insert) and the fragment filled in with Klenow fragment and dNTPs. Next, the fragment was gel purified, then digested with NdeI and inserted in pT7-Ea that had been digested with NdeI and BamHI (filled

with Klenow) and phosphatase treated. The new plasmid was designated pT7-Ea-hPMS134. The following strategy, similar to that described above to clone human PMS134, was used to construct an expression vector for the human related gene PMSR3. First, the hPMSR3 fragment was amplified by PCR to introduce two restriction sites, an NdeI restriction site at the 5'-end and an Eco RI site at the 3'-end of the fragment. The 5'-oligonucleotide that was used for PCR has the following structure: 5'-ACG CAT ATG TGT CCT TGG CGG CCT AGA-3' that includes the NdeI restriction site CAT ATG. The 3'-oligonucleotide used for PCR has the following structure: 5'-GAA TTC TTA TTA CGT AGA ATC GAG ACC GAG GAG AGG GTT AGG GAT AGG CTT ACC CAT GTG TGA TGT TTC AGA GCT-3' that includes an EcoRI site GAA TTC and the V5 epitope to allow for antibody detection. The plasmid that contained human PMSR3 in pBluescript SK (19) was used as the PCR target with the hPMS2-specific oligonucleotides above. Following 25 cycles of PCR (95^0C for 1 minute, 55^0C for 1 minute, 72^0C for 1.5 minutes for 25 cycles followed by 3 minutes at 72^0C). The PCR fragment was purified by gel electrophoresis and cloned into pTA2.1 (InVitrogen) by standard cloning methods (Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Edition, 2001), creating the plasmid pTA2.1-hR3. pTA2.1-hR3 was next digested with the restriction enzyme EcoRI to release the insert (there are two EcoRI restriction sites in the multiple cloning site of pTA2.1 that flank the insert) and the fragment filled in with Klenow fragment and dNTPs. Then, the fragment was gel purified, then digested with NdeI and inserted in pT7-Ea that had been digested with NdeI and BamHI (filled with Klenow) and phosphatase treated. The new plasmid was designated pT7-Ea-hR3.

BL21 cells harbor an additional expression vector for the lysozyme protein, which has been demonstrated to bind to the T7 polymerase in situ; this results in a bacterial strain that has very low levels of T7 polymerase

expression. However, upon addition of the inducer IPTG, the cells express high-levels of T7 polymerase due to the IPTG-inducible element that drives expression of the polymerase that is resident within the genome of the BL21 cells (30). The BL21 cells are chloramphenicol resistant due to the plasmid that expresses lysozyme within the cell. To introduce the pT7-hPMS134 or the pT7-hPMSR3 genes into BL21 cells, the cells were made competent by incubating the cells in ice cold 50mM $CaCl_2$ for 20 minutes, followed by concentrating the cells and adding super-coiled plasmid DNA as desecribe (Maniatis, T.et.al. Cold Spring Harbor Laboratory Press, Third Edition, 2001). Ampicillin resistant BL21 were selected on LB-agar plates [5% yeast extract, 10% bactotryptone, 5% NaCl, 1.5% bactoagar , pH 7.0 (Difco)] plates containing 25 μg/ml chloramphenicol and 100μg/ml ampicillin. The next day, bacterial colonies were picked and analyzed for vectors containing an intact pTACPMS134 or pTAC empty vector by restriction endonuclease digestion and sequence analysis.

In addition to constructing a V5-epitope tagged PMS134 construct we also constructed and tested a non-epitope tagged version. This was prepared to demonstrate that the simple fact of epitope tagging the construct did not result in alteration of the dominant-negative phenotype that PMS134 has on mismatch repair activity. For these studies, a BamHI restriction fragment containing the hPMS134 cDNA was filled-in with Klenow fragment and then sub-cloned into a Klenow-filled blunt-ended NdeI-XhoI site of the pTACLAC expression vector, which contains the isopropylthio-β-galactosidase (IPTG)-inducible bacterial TAC promoter and ampicillin resistance gene as selectable marker. The NdeI-XhoI cloning site is flanked by the TACLAC promoter that contains the LacI repressor site followed by a Shine Dalgarno ribosome-binding site at the 5' flanking region and the T1T2 ribosomal RNA terminator in the 3' flanking region. The TACLAC vector also contains the LacI gene, which is constitutively expressed by the TAC promoter.

DH10B bacterial cells containing the pBCSK vector (Stratagene), which constitutively expresses the β-galactosidase gene and contains the chloramphenicol resistance marker for selection, were made competent via the $CaCl_2$ method (Maniatis, T.et.al. Cold Spring Harbor Laboratory Press, 1982). This vector turns bacterial cells blue when grown in the presence of IPTG and X-gal that aids in the detection of bacterial colonies. Competent cells were transfected with the pTAC empty vector or the pTAC*PMS134* vector following the heat-shock protocol. Transfected cultures were plated onto LB-agar [5% yeast extract, 10% bactotryptone, 5% NaCl, 1.5% bactoagar , pH 7.0 (Difco)] plates containing 25 μg/ml chloramphenicol and 100μg/ml ampicillin. The next day, bacterial colonies were picked and analyzed for vectors containing an intact pTACPMS134 or pTAC empty vector by restriction endonuclease digestion and sequence analysis. Ten clones of each bacteria containing correct empty or PMS134 inserts were then grown to confluence overnight in LB media (5% yeast extract, 10% bactotryptone, 5% NaCl, pH 7.0) containing 10 μg/ml chloramphenicol and 50 μg/ml ampicillin. The next day TAC empty or pTAC*PMS134* cultures were diluted 1:4 in LB medium plus 50μM IPTG (Gold Biotechnology) and cultures were grown for 12 and 24 hours at 37°C. After incubation, 50μl aliquots were taken from each culture and added to 150μls of 2X SDS buffer and cultures were analyzed for PMS134 protein expression by western blot.

Western blots were carried out as follows. 50μls of each PMS134 or empty vector culture was directly lysed in 2X lysis buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 M 2-mercaptoethanol, 0.001% bromophenol blue) and samples were boiled for 5 minutes. Lysate proteins were separated by electrophoresis on 4-20% Tris glycine gels (Novex). Gels were electroblotted onto Immobilon-P (Millipore) in 48 mM Tris base, 40 mM glycine, 0.0375% SDS, 20% methanol and blocked overnight

at 4°C in Tris-buffered saline plus 0.05% Tween-20 and 5% condensed milk. Filters were probed with a rabbit polyclonal antibody generated against the N-terminus of the human PMS2 polypeptide (Santa Cruz), which is able to recognize the PMS134 polypeptide (31), followed by a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody. After incubation with the secondary antibody, blots are developed using chemiluminescence (Pierce) and exposed to film to measure PMS134 expression.

As shown in Figure 1, a robust expression of PMS134 could be detected in bacterial cells containing pTACPMS134 (lane 2) in contrast to cells expressing empty vector (lane 1), which had no signal.

For induction of PMS134 and PMSR3 in BL21 cells, the pT7-Ea-hPMS134 or the pT7-Ea-hPMSR3 cells were induced with 50μM IPTG for 12 and 24 hours. Cell lysates were prepared and analyzed by western blot listed above using either the N-terminal PMS2 antibody to detect the PMS134 containing cells or the antiV5-horseradish peroxidase conjugated monoclonal antibody (InVitrogen) to detect the PMS134V5 and PMSR3V5 polypeptides. Figure 2 shows the expression of PMS134V5 and PMSR3V5 before (-) lanes and after IPTG (+) lanes induction.

Example 2: Generation of hypermutable bacteria with inducible dominant negative alleles of mismatch repair genes

Bacterial clones expressing the *PMS134* or the empty vector were grown in liquid culture for 24 hr at 37°C in the presence of 10 μg/ml chloramphenicol and 50 μg/ml ampicillin plus 50μM IPTG. The next day, cultures were diluted 1:10 in medium containing 50μM IPTG plus ampicillin/chloramphenicol (AC) or ampicillin/chloramphenicol plus 25 μg/ml kanamycin (ACK) and cultures were grown for 18 hr at 37°C. The following day, a 0.1 μl aliquot (2 μl diluted in 1000 μl of LB medium and

used 50 µl for plating) of cells grown in AC medium were plated on LB-agar plates containing 40 µg/ml of 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal) plus 100µg/ml ampicillin (AMP), while a 1 µl aliquot (1 µl diluted in 100 µl of LB medium and used 100 µl for plating) of cells grown in ACK medium were plated on LB-agar plates containing X-gal and 50µg/ml kanamycin (KAN). Plates were incubated for 18 hours at 37°C. The results from these studies show that cells expressing the *PMS134* were able to increase hypermutation in the genome of the DH10B bacterial strain which resulted in the production of siblings that exhibit new biological traits such as KAN resistance (Figure 3).

Kanamycin-resistant assays using BL21 cells expressing the V5-tagged or untagged PMS134 or PMSR3 polypeptides were carried out as described above. BL21 bacterial cells that harbor the empty vector, pT7-PMS134 or pT7-PMSR3 were grown overnight in LB supplemented with 100ug/ml ampicillin. The overnight cultures were diluted 1:100 into fresh ampicillin containing medium and grown for 2.5 hours at 37^0C with continuous shaking. When the cells reached an optical density (OD) of 0.6, measured at 600nm, IPTG was added to each culture to a final concentration of 0.5mM. Cells were incubated for 24, and 48 hours; at those time points cells were removed for SDS-PAGE analysis and plating (see above). BL21/pT7 (empty vector), BL21/pT7-PMS134, and BL21/pT7-R3 cells were plated onto LB plates, LB plates that contained 100ug/ml ampicillin, and plates that contain 50ug/ml Kanamycin. The equivalent of 1 x 10^7 cells/plate were spread onto the plates. BL21 cells that harbor the empty vector are capable of growth on LB plates as well as LB plates that contain 100ug/ml ampicillin; that is as expected since the pT7 expression vector renders the cells ampicillin resistant. The vector only control is not capable of growth on Kanamycin. After 24hr IPTG-induction PMS134 or PMSR3 cells

had a significant number of KAN resistant cells while none were observed in BL21 parental cells grown under similar conditions (Figure 4). Moreover, BL21 cells containing the PMS134 or PMSR3 genes under non-IPTG-induced conditions failed to produce any KAN resistant clones demonstrating the need for expression of the PMS polypeptides for hypermutability. A summary outlining the data and number of Kanamycin resistant bacterial clones is provided in TABLE 1.

TABLE 1. Generation of Kanamycin resistant clones via MMR deficiency

STRAIN	# CELLS SEEDED	AMP^R colonies	KAN^R colonies	FREQUENCY
DH10B VEC	50,000	62,000	0	0
DH10B PMS134	50,000	43,146	23	5.3×10^{-4}
BL21 VEC	500,000	520,800	0	0
BL21 T7-Ea-PMS134V5	500,000	450,000	2,245	4.9×10^{-3}
BL21 T7-Ea-PMSR3V5	500,000	500,000	1,535	3.1×10^{-3}

These data demonstrate and enable the proof-of-concept that the use of the dominant negative MMR genes is a viable approach to creating hypermutable bacteria that can lead to the generation of phenotypically diverse offspring when put under selective conditions.

Using the same protocol as listed above and the same cloning strategy, a truncated PMS2 homolog from the *Arabidopsis thaliana* plant **(reference the PLANT patent),** which was cloned by degenerate PCR from an *Arabidopsis thaliana* cDNA library

(Strategene), was found to give a similar enhancement of genetic hypermutability in DH5alpha bacteria Figure 5. For detection purposes, we fused a FLAG epitope to the C-terminus of the PMS134 polypeptide using PCR and an antisense primer directed to the 134 codon region of the *Arabidopsis* PMS2 homolog followed by a FLAG epitope and 2 termination codons. The resultant fusion was termed ATPMS134-flag. The AT PMS134-flag gene was then cloned into the IPTG-inducible TACLAC expression vector and transfected into DH5alpha cells. Western blot of bacteria transfected with an IPTG-inducible expression vector carrying a truncated version (codons 1-134) of the Arabidopsis thaliana PMS2 homolog using the anti-FLAG antibody demonstrated the inducibility and steady-state protein levels of the chimeric gene. Figure 5A shows the western blot containing protein from an untransfected cell (lane 1) and a bacterial clone expressing the Arabidopsis PMS2-134 truncated protein (lane 2). Following the mutagenesis protocol described above, bacterial cells expressing the ATPMS134 protein were found to have an increase in the number of KAN resistant cells (12 clones) in contrast to cells expressing the empty vector that yielded no KAN resistant clone.

Bacterial cells such as the pT7-PMS134 and pT7-R3 harboring BL21 cells; the TACLACPMS134 DH10B; the TACLACMLH1 DH10B cells; or the TACLAC-ATPMS134flag DH5alpha cells are capable of growth on LB, LB/ampicillin and LB/KAN plates because the cells have acquired mutations within their genome that render the cell drug resistant. Cells that express dominant negative MMR genes have altered the mismatch control pathway of the microbe, presumably altering a gene or a set of genes that control resistance to kanamycin. A new output trait, Kanamycin-resistance, is generated by expression of the dominant negative MMR

gene in these cells. These data demonstrate the ability of dominant negative MMR genes to produce hypermutability across a wide array of bacterial strains to produce new output traits such as Kanamycin resistance.

EXAMPLE 3: Dominant negative MMR genes can produce new genetic variants and commercially viable output traits in prokaryotic organisms.

The data presented in EXAMPLE 2 show the ability to generate genetic alterations and new phenotypes in bacterial strains expressing dominant negative MMR genes. In this EXAMPLE we teach the utility of this method to create prokaryotic strains with commercially relevant output traits.

GENERATION OF HEAT-RESISTANT PRODUCER STRAINS.

One example of commercial utility is the generation of heat-resistant recombinant protein producer strains. In the scalable process of recombinant manufacturing, large-scale fermentation of prokaryotes results in the generation of heat, which leads to suboptimal growth conditions for the producer strain and thus resulting in lower recombinant protein yields. In order to circumvent this problem, we employed the use of DH10B bacteria containing the inducible TACLACPMS134 gene. Briefly, cells were grown in 5 ml LB shake flasks containing ampicillin and IPTG-induced for 0, 24 and 48hrs at 37C. Cultures were harvested and then incubated at 100C for 0, 1 or 10 minutes (times at which 100% of the wild-type strain perishes) and 100 µl aliquots (equivalent to 250,000 cells) were plated onto LB agar plates containing ampicillin to identify heat

resistant clones. Table 2 shows a typical experiment whereby cells containing the TACLACPMS134 gene generated a significant number of heat-resistant clones after 48 hours of PMS134 induction and hypermutation via MMR blockade. No or a few clones were observed in the uninduced or 24hr induced conditions respectively suggesting the needs for multiple rounds of genetic mutation to produce genes that are capable of allowing bacteria to survive under harsh conditions. Similar results were observed with other dominant negative mutants such as the PMSR2, PMSR3, and the human MLH1 proteins (not shown).

TABLE 2. Generation of heat-resistant clones via MMR deficiency

Treatment	Heated 0 min	Heated 1 min	Heated 10 min
TACLACVEC 0 hr IPTG	250,000 +/- 7,500	0	0
TACLACPMS134 0 hr IPTG	265,000 +/- 2,000	0	0
TACLACVEC 24 hr IPTG	274,000 +/- 12,000	1 +/- 0	0
TACLACPMS134 24 hr IPTG	240,000 +/- 9,400	5 +/- 2	0
TACLACVEC 48 hr IPTG	256,000 +/- 12,000	0	0
TACLACPMS134 48 hr IPTG	252,000 +/- 14,000	65 +/- 8	3 +/- 1

GENERATION OF HIGH RECOMBINANT PROTEIN PRODUCER STRAINS.

Next, we tested the ability of bacteria expressing dominant negative MMR genes to produce sublclones with enhanced recombinant protein production. In these experiments again we employed the DH10B cells containing the TACLACPMS134 inducible vector plus the pTLACZ vector, which constitutively

expresses the β-galactosidase gene. Analysis of individual clones containing the TACLACPMS134 and pTLACZ vector typically produces 10-20μg/ml of LACZ protein via shake flask fermentation after IPTG induction for 24 hours. To test the hypothesis that high recombinant producer strains can be generated by decreased MMR in bacterial strains, we induced the TACLACPMS134-pTLACZ cells for 48 hours with IPTG as described above. We then diluted the culture 1:50 in LB medium, grew the strain for 24 hours, and plated 10 μls of culture (diluted in 300 μls of LB) onto LB amp-XGAL plates to identify candidate clones that produce robust levels of recombinant LACZ protein. As a control, uninduced cells were treated similarly and plated onto LB amp-XGAL plates. Analysis of the plates revealed a number of bacterial colonies exhibiting a number of clones with an intense BLUE staining in the TACLACPMS134/pTLACZ cells induced with IPTG but none were observed in uninduced clones (Figure 6). To confirm that these clones produced an enhanced level of LACZ, we expanded 2 clones with an average BLUE stain (BGAL-C1 and BGAL-C2) and 10 clones with a robust BLUE staining (BGAL-MOR1 to BGAL-MOR10). We grew all clones in LB AMP for 24 hours without IPTG and replated the clones. Six out of ten BGAL-MOR clones resulted in a more robust β-gal stain *in situ* as compared to control "average" cells (BGALC1 and C2). We next performed a more quantitative assay using a β-gal ELISA assay. Briefly, 2 mls of cell centrifuged at 10,000gs for 10 minutes and resuspended in 0.5 mls of 0.25M Tris, pH 7.5 plus 0.0001% Tween-20. Cells were freeze-thawed 4X's and vortexed for 4 minutes at room temperature. Lysates were cleared of debris by centrifugation and supernatants were collected. Protein extracts were quantified for total protein using the Bradford assay (BioRad) as described by the manufacturer. Plate ELISAs were

carried out by coating 96 well maxisorb (NUNC) plates with 0.1 mls of a 1 μg/ml (diluted in PBS pH7.0) bacterial extract solution and a dose range of recombinant β-GAL (Sigma) from 0.001 to 10 mg/ml. All samples were plated in triplicates. Plates were coated for 2 hours, washed 2 times with PBS and blocked with 0.2mls of PBS plus 5% powdered milk for 30 minutes. Next, plates were washed once with PBS and incubated with an anti-β-galactosidase monoclonal antibody that recognizes both native and denatured forms (Sigma) for 2 hours. Plates were then washed 3 times with PBS and incubated with 0.1 mls of an anti-mouse horseradish peroxidase conjugated antibody for 1 hour at room temperature. Plates were washed 3 times with PBS and incubated with TMB ELISA substrate (BioRad) for 15 to 30 minutes. Reactions were stopped with 0.1N H_2SO_4 and read on a BioRAD plate reader at 415nm. The control clones produced roughly 9 and 13 μgs /ml of β-gal while BGAL-MOR clones 2, 3 and 9 produced 106, 82 and 143 μgs /ml of β-gal. To determine if reason that these clones produced more β-gal was due to mutations in the plasmid promoter elements, we isolated the pTLACZ plasmid and retransfected it into DH10B cells as described above. *In situ* analysis found the resultant clones to produce similar amount of β-gal as that of the control. These data suggest that the BGAL-MOR 2, 3, and 9 hosts had alterations, which results in elevated expression and/or stability of recombinant proteins.

To determine if the enhanced *in situ* β-gal expression that was observed in BGAL-MOR clones 1, 5, and 6, which did not appear to have enhanced β-gal protein levels (had less than 15 μg/ml as determined by ELISA) was authentic, we performed a more quantitative assay on these lines plus the BGAL-MOR 9, the BGALC1 and C2 lines as control. Cells containing an empty vector

(without a LACZ gene) were used as negative control. To measure β-gal activity, we employed a colorimetric β-gal substrate assay using CPRG (Roche) as described (31). Briefly, 5 μgs of protein extract isolated for ELISA analysis (described above) were analyzed using a plate assay. Protein was added to buffer containing 45 mM 2-mercaptoethanol, 1mM $MgCl_2$, 0.1 M $NaPO_4$ and 0.6 mg/ml Chlorophenol red-β-D-galactopyranoside (CPRG, Roche). Reactions were incubated for 1 hour, terminated by the addition of 0.5 M Na_2CO_3, and analyzed by spectrophotometry at 576 nm in a BioRad plate reader. Analysis of these extracts confirmed our *in situ* data that these cells did have increased β-gal activity (TABLE 3).

TABLE 3. Generation of bacterial clones with increased β-gal enzymatic activity via MMR deficiency.

Clone	β-gal protein (μg/ml)	β-gal activity (O.D. 576)
BGAL-C1	9	0.413 +/- .092
BGAL-C2	13	0.393 +/- .105
BGAL-MOR1	14	0.899 +/- .134
BGAL-MOR5	13	0.952 +/- .133
BGAL-MOR6	15	0.923 +/- .100
BGAL-MOR9	143	0.987 +/- .106
Empty vector	-	0.132 +/- .036

Because there was no observable increase in the amount of β-gal protein one likely hypothesis is that the β-gal gene structure was mutated during the hypermutability growth stage and now produces a

more active enzyme. Sequence analysis confirms that this may be the reason for enhanced activity in a subset of clones.

Together, these data demonstrate the ability to produce genetically altered prokaryotic host strains using dominant negative MMR genes to generate commercially valuable output traits such as high recombinant protein producer lines and structurally altered enzymes with enhanced activities.

EXAMPLE 4: Mutations in the host genome generated by defective MMR are genetically stable

As described in EXAMPLE 2 and 3, manipulation of the MMR pathway in microbes results in alterations within the host genome and the ability to select for a novel output traits. It is important that the mutations introduced as a result of defective MMR is genetically stable and passed on to daughter cells once a desired output pathway is established. To determine the genetic stability of mutations introduced into the microbial genome the following experiment was performed. Five independent colonies from pT7-PMS134 and pT7-PMSR3 that are kanamycin resistance were grown overnight from an isolated colony in 5 ml of LB. Next, 1μL of the overnight culture from these cultures were inoculated into another 5 mL of LB and grown overnight to saturation. Under these growth conditions the microbial cells have divided over 20 generations. Therefore, if the new output trait generated by alteration of MMR is unstable, the cells should "revert" back from kanamycin resistance to kanamycin sensitivity. Cells were plated onto LB plates and incubated overnight at 37^0C. Next, the colonies (about 1,000/plate) were replica plated to LB, LB^{amp100}, and LB^{kan50} plates and incubated at 37^0C overnight. Analysis of clones from these studies reveal that a

strict correlation occurs with loss of dominant negative MMR expression and phenotype stability. No loss of KAN resistant clones generated in Example 3 were observed when cells were grown in the absence of IPTG (not expressing PMS134), while 5 revertants out of 1200 were observed in clones the were continually grown in IPTG (express PMS134). Extended culturing of cells and replica plating found no reversions of KAN resistance in cultures grown in the absence of IPTG, which produce no PMS134 as determined by western blot (data not shown).

These data demonstrate the utility of employing inducible expression systems and dominant negative MMR genes in prokaryotes to generate genetically altered strains for commercial applications such as but not limited to enhanced recombinant manufacturing and biotransformation that can then in turn be restored to a genetically stable host with a "fixed" new genotype that is suitable for commercial processes.

EXAMPLE 5: Enhanced Generation of MMR-Defective Bacteria and Chemical Mutagens for the Generation of New Output Traits

It has been previously documented that MMR deficiency yields to increased mutation frequency and increased resistance to toxic effects of chemical mutagens (CM) and their respective analogues such as but not limited to those as: ethidium bromide, EMS, MNNG, MNU, Tamoxifen, 8-Hydroxyguanine, as well as others listed but not limited to in publications by: Khromov-Borisov, N.N., et.al. (Mutat. Res. 430:55-74, 1999); Ohe, T., et.al. (Mutat. Res. 429:189-199, 1999); Hour, T.C. et.al. (Food Chem. Toxicol. 37:569-579, 1999); Hrelia, P., et.al. (Chem. Biol. Interact. 118:99-111, 1999); Garganta, F., et.al. (Environ. Mol. Mutagen. 33:75-85, 1999);

Ukawa-Ishikawa S., et.al. (Mutat. Res. 412:99-107, 1998); www.ehs.utah.edu/ohh/mutagens, etc. To demonstrate the ability of CMs to increase the mutation frequency in MMR defective bacterial cells, we exposed T7-PMS134 BL21 cells to CMs.

T7-PMS134 cells and empty vector control cells were grown with IPTG for 48 hours and then diluted 1: 50 in LB plus IPTG and increasing amounts of ethyl methane sulfonate (EMS) from 0, 1, 10, 50, 100, and 200 μM. 10 μL aliquots of culture (diluted in 300 μl LB) were plated out on LB agar plus ampicillin plates and grown overnight at 37C. The next day plates were analyzed for cell viablility as determined by colony formation. Analysis found that while no significant difference in colony number was observed between the pT7-PMS134 and control at the 0, 1 , or 10 uM concentrations (all had > 1000 colonies), the number of control cells were reduced to 30 and 0 at the 50 and 100 μM concentrations, respectively. No difference was observed in the pT7-PMS134 cells treated with 0, 1, 10 or 50 μM, while a 3 fold reduction was observed in cultures treated with 100 μM EMS. The 200 μM treatment was toxic for both lines. These data demonstrate the ability of MMR deficiency to protect prokaryotes against the toxic effects of DNA akylating agents and provides a means to generate a wider range of mutations that can lead to an increased number of genetic variations and an increase in the number of new biochemical activities within host proteins to produce new output traits for commercial applications.

To confirm that MMR deficient bacterial cells treated with CM can result in an increased mutation rate and produce a greater number of variants, we cultured pT7-PMS134 cells and empty vector controls in the presence of IPTG for 48 hours, followed by dilution and regrowth in 25μM EMS for 24 hours as described above. Cells were plated out on 100 mM petri dishes containing amplicillin or KAN and scored for KAN resistance. Analysis revealed that an 11-fold increase in the generation of KAN

resistant cells were found in pT7-Ea-PMS134V5 cells in contrast to control cells.

These data demonstrate the use of employing a regulated dominant negative MMR system plus chemical mutagens to produce enhanced numbers of genetically altered prokaryotic strains that can be selected for new output traits. This methods is now useful generating such organims for commercial applications such as but not limited to recombinant manufacturing, biotransformation, and altered biochemicals (biotransformation) with enhanced activities for manufacturing purposes and gene discovery for pharmaceutical compound development.

EXAMPLE 6: Alternative Methods to Inhibition of Bacterial MMR Activity

The inhibition of MMR activity in a host organism can be achieved by introducing a dominant negative allele as shown in EXAMPLES 2 and 3. This application also teaches us the use of using regulated systems to control MMR in prokaryotes to generate genetic diversity and output traits for commercial applications. Other ways to regulate the suppression of MMR activity of a host is by using genetic recombination to knock out alleles of a MMR gene that can be spliced out such after selection using a system such as the CRE-Lox system; 2) blocking MMR protein dimerization with other subunits (which is required for activity) by the introduction of polypeptides or antibodies into the host via transfection methods routinely used by those skilled in the art; or 3) decreasing the expression of a MMR gene using anti-sense oligonucleotides.

MMR gene knockouts. We intend to generate disrupted targeting vectors of a particular MMR gene and introduce it into the genome of bacteria using methods standard in the art. Bacteria exhibiting hypermutability will be useful to produce genetically diverse offspring for commercial applications.

Bacteria will be confirmed to have lost the expression of the MMR gene using standard northern and biochemical techniques (as described in reference 32). MMR gene loci can be knocked out, strains selected for new output traits and MMR restored by introducing a wildtype MMR gene to complement the KO locus. Other strategies include using KO vectors that can target a MMR gene locus, select for host output traits and then have the KO vector "spliced" from the genome after strain generation. This process could be performed using systems such as but not limited to CRE-Lox.

Blocking peptides. MMR subunits (MutS and MutL proteins) interact to form active MMR complexes. Peptides are able to specifically inhibit the binding of two proteins by competitive inhibition. The use of peptides or antibodies to conserved domains of a particular MMR gene can be introduced into prokaryotic cells using lipid transfer methods that are standard in the art. Bacteria will be confirmed to have lost the expression of the MMR gene using standard northern and biochemical techniques (as described in reference 32). Bacteria exhibiting hypermutability will be useful to produce genetically diverse sibs for commercial applications.

Discussion

The results described above will lead to several conclusions. Yhe expression of dominant negative MMR proteins results in an increase in hypermutability in bacteria. This activity is due to the inhibition of MMR biochemical activity in these hosts. This method provides a claim for use of dominant negative MMR genes and their encoded products for the creation of hypermutable bacteria to produce new output traits for commercial applications.

EXAMPLES OF MMR GENES AND ENCODED POLYPEPTIDES

Yeast MLH1 cDNA (accession number U07187)

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   1 aaataggaat gtgatacctt ctattgcatg caaagatagt gtaggaggcg ctgctattgc
  61 caaagacttt tgagaccgct tgctgtttca ttatagttga ggagttctcg aagacgagaa
 121 attagcagtt ttcggtgttt agtaatcgcg ctagcatgct aggacaattt aactgcaaaa
 181 ttttgatacg atagtgatag taaatggaag gtaaaaataa catagaccta tcaataagca
 241 atgtctctca gaataaaagc acttgatgca tcagtggtta acaaaattgc tgcaggtgag
 301 atcataatat cccccgtaaa tgctctcaaa gaaatgatgg agaattccat cgatgcgaat
 361 gctacaatga ttgatattct agtcaaggaa ggaggaatta aggtacttca aataacagat
 421 aacggatctg gaattaataa agcagacctg ccaatcttat gtgagcgatt cacgacgtcc
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Yeast MLH1 protein (accession number U07187)

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Mouse PMS2 protein

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859

Mouse PMS2 cDNA

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human PMS2 protein
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ILKPDLRIVF VHNKAVIWQK SRVSDHKMAL MSVLGTAVMN NMESFQYHSE ESQIYLSGFL 240
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VPTADVDVNL TPDKSQVLLQ NKESVLIALE NLMTTCYGPL PSTNSYENNK TDVSAADIVL 360
SKTAETDVLF NKVESSGKNY SNVDTSVIPF QNDMHNDESG KNTDDCLNHQ ISIGDFGYGH 420
CSSEISNIDK NTKNAFQDIS MSNVSWENSQ TEYSKTCFIS SVKHTQSENG NKDHIDESGE 480
NEEEAGLENS SEISADEWSR GNILKNSVGE NIEPVKILVP EKSLPCKVSN NNYPIPEQMN 540
LNEDSCNKKS NVIDNKSGKV TAYDLLSNRV IKKPMSASAL FVQDHRPQFL IENPKTSLED 600
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Human PMS2 cDNA

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2771

human PMS1 protein
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932

Human PMS1 cDNA
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ctgacttgtt tttatattga aaaaagttcc acgtattgta gaaaacgtaa ataaactaat 3060
aac 3063

human MSH2 protein

MAVQPKETLQ LESAAEVGFV RFFQGMPEKP TTTVRLFDRG DFYTAHGEDA LLAAREVFKT 60
QGVIKYMGPA GAKNLQSVVL SKMNFESFVK DLLLVRQYRV EVYKNRAGNK ASKENDWYLA 120
YKASPGNLSQ FEDILFGNND MSASIGVVGV KMSAVDGQRQ VGVGYVDSIQ RKLGLCEFPD 180
NDQFSNLEAL LIQIGPKECV LPGGETAGDM GKLRQIIQRG GILITERKKA DFSTKDIYQD 240
LNRLLKGKKG EQMNSAVLPE MENQVAVSSL SAVIKFLELL SDDSNFGQFE LTTFDFSQYM 300
KLDIAAVRAL NLFQGSVEDT TGSQSLAALL NKCKTPQGQR LVNQWIKQPL MDKNRIEERL 360
NLVEAFVEDA ELRQTLQEDL LRRFPDLNRL AKKFQRQAAN LQDCYRLYQG INQLPNVIQA 420
LEKHEGKHQK LLLAVFVTPL TDLRSDFSKF QEMIETTLDM DQVENHEFLV KPSFDPNLSE 480
LREIMNDLEK KMQSTLISAA RDLGLDPGKQ IKLDSSAQFG YYFRVTCKEE KVLRNNKNFS 540
TVDIQKNGVK FTNSKLTSLN EEYTKNKTEY EEAQDAIVKE IVNISSGYVE PMQTLNDVLA 600
QLDAVVSFAH VSNGAPVPYV RPAILEKGQG RIILKASRHA CVEVQDEIAF IPNDVYFEKD 660
KQMFHIITGP NMGGKSTYIR QTGVIVLMAQ IGCFVPCESA EVSIVDCILA RVGAGDSQLK 720
GVSTFMAEML ETASILRSAT KDSLIIIDEL GRGTSTYDGF GLAWAISEYI ATKIGAFCMF 780
ATHFHELTAL ANQIPTVNNL HVTALTTEET LTMLYQVKKG VCDQSFGIHV AELANFPKHV 840
IECAKQKALE LEEFQYIGES QGYDIMEPAA KKCYLEREQG EKIIQEFLSK VKQMPFTEMS 900
EENITIKLKQ LKAEVIAKNN SFVNEIISRI KVTT 934

Human MSH2 cDNA

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ataaataaaa tcatgtagtt tgtgg
3145

human MLH1 protein
MSFVAGVIRR LDETVVNRIA AGEVIQRPAN AIKEMIENCL DAKSTSIQVI VKEGGLKLIQ 60
IQDNGTGIRK EDLDIVCERF TTSKLQSFED LASISTYGFR GEALASISHV AHVTITTKTA 120
DGKCAYRASY SDGKLKAPPK PCAGNQGTQI TVEDLFYNIA TRRKALKNPS EEYGKILEVV 180
GRYSVHNAGI SFSVKKQGET VADVRTLPNA STVDNIRSIF GNAVSRELIE IGCEDKTLAF 240
KMNGYISNAN YSVKKCIFLL FINHRLVEST SLRKAIETVY AAYLPKNTHP FLYLSLEISP 300
QNVDVNVHPT KHEVHFLHEE SILERVQQHI ESKLLGSNSS RMYFTQTLLP GLAGPSGEMV 360
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DGPKEGLAEY IVEFLKKKAE MLADYFSLEI DEEGNLIGLP LLIDNYVPPL EGLPIFILRL 660
ATEVNWDEEK ECFESLSKEC AMFYSIRKQY ISEESTLSGQ QSEVPGSIPN SWKWTVEHIV 720
YKALRSHILP PKHFTEDGNI LQLANLPDLY KVFERC 756

Human MLH1 cDNA
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hPMS2-134 protein

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IKAVDAPVMA MKYYTSKINS HEDLENLTTY GFRGEALGSI CCIAEVLITT RTAADNFSTQ 120
YVLDGSGHIL SQK 133

hPMS2-134 cDNA

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acttga 426

hMSH6 (human cDNA) ACCESSION U28946

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hPMSR2 (human cDNA) ACCESSION U38964

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  61 ttggggagcc agtacatgca ggtgggctcc acacggagag gggcgcagac ccggtgacag
 121 ggctttacct ggtacatcgg catggcgcaa ccaaagcaag agagggtggc gcgtgccaga
 181 caccaacggt cggaaaccgc cagacaccaa cggtcggaaa ccgccaagac accaacgctc
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hPMSR2 (human protein) ACCESSION U38964

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HPMSR3 (human cDNA) ACCESSION U38979

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 661 gcccccagaa ggacacccgc ctggatttgc cccgtagccc ggcccgggcc cctcgggagc
 721 agaacagcct tggtgaggtg gacaggaggg gacctcgcga gcagacgcgc gcgccagcga
 781 cagcagcccc gccccggcct ctcgggagcc ggggggcaga ggctgcggag ccccaggagg
 841 gtctatcagc cacagtctct gcatgtttcc aagagcaaca ggaaatgaac acattgcagg
 901 ggccagtgtc attcaaagat gtggctgtgg atttcaccca ggaggagtgg cggcaactgg
 961 accctgatga gaagatagca tacggggatg tgatgttgga gaactacagc catctagttt
1021 ctgtggggta tgattatcac caagccaaac atcatcatgg agtggaggtg aaggaagtgg
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1141 ctgctaaggc catcaaacct attgatcgga agtcagtcca tcagatttgc tctgggccag
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1381 cacacatgta agattcaaga gtttgccgac ctaactgaag ttgaaacttt cggttttcag
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1561 ccctaccccc accccagagg gaccacagtc agcgtgaagc agttattttc tacgctacct
1621 gtgcgccata aggaatttca aaggaatatt aagaagacgt gcctgcttcc ccttcgcctt
1681 ctgccgtgat tgtcagtttc ctgaggcctc cccagccatg cttcctgtac agcctgcaga
1741 actgtgagtc aattaaacct cttttcttca taaattaaaa aaaaa
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hPMSR3 (human protein) ACCESSION U38979

```
MCPWRPRLGRRCMVSPREADLGPQKDTRLDLPRSPARAPREQNS
LGEVDRRGPREQTRAPATAAPPRPLGSRGAEAAEPQEGLSATVSACFQEQQEMNTLQG
PVSFKDVAVDFTQEEWRQLDPDEKIAYGDVMLENYSHLVSVGYDYHQAKHHHGVEVKE
VEQGEEPWIMEGEFPCQHSPEPAKAIKPIDRKSVHQICSGPVVLSLSTAVKELVENSL
DAGATNIDLKLKDYGVDLIEVSDNGCGVEEENFEGLISFSSETSHM"
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hPMSL9 (human cDNA) ACCESSION NM_005395

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 121 gagcagaaca gccttggtga ggtggacagg aggggacctc gcgagcagac gcgcgcgcca
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 301 caggggccag tgtcattcaa agatgtggct gtggatttca cccaggagga gtggcggcaa
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 421 gtttctgtgg ggtatgatta tcaccaagcc aaacatcatc atggagtgga ggtgaaggaa
 481 gtggagcagg gagaggagcc gtggataatg gaaggtgaat ttccatgtca acatagtcca
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 601 ccagtggtac tgagtctaag cactgcagtg aaggagttag tagaaaacag tctggatgct
 661 ggtgccacta atattgatct aaagcttaag gactatggag tggatctcat tgaagtttca
 721 gacaatggat gtggggtaga agaagaaaac tttgaaggct taatctcttt cagctctgaa
 781 acatcacaca tgtaa
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hPMSL9 (human protein) ACCESSION NM_005395

```
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DAGATNIDLKLKDYGVDLIEVSDNGCGVEEENFEGLISFSSETSHM"
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